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Active Escape of *Orientia tsutsugamushi* from Cellular Autophagy

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***Orientia tsutsugamushi*, the causative agent of scrub typhus, is an obligate intracellular pathogen. After entry into host cells, the bacterium rapidly escapes from the endosomal pathway and replicates in the cytosol of eukaryotic host cells. Here we show that *O. tsutsugamushi* infection efficiently promotes cellular autophagy, a cell-autonomous defense mechanism of innate immunity. However, most of the internalized bacteria barely colocalized with the induced autophagosomes, even when stimulated with rapamycin, a chemical inducer of autophagy. Treatment of infected cells with tetracycline suppressed bacterial evasion from autophagy and facilitated *O. tsutsugamushi* targeting to autophagosomes, suggesting that the intracellular pathogen may be equipped with a bacterial factor or factors that block autophagic recognition. Finally, we also found that chemical modulators of cellular autophagy or genetic knockout of the *atg3* gene does not significantly affect the intracellular growth of *O. tsutsugamushi in vitro*. These results suggest that *O. tsutsugamushi* has evolved to block autophagic microbicidal defense by evading autophagic recognition even though it activates the autophagy pathway during the early phase of infection.**

Autophagy is an evolutionarily conserved and regulated intracellular catabolic mechanism that mediates the degradation of cytosolic components, including protein complexes and damaged organelles, in a lysosome-dependent manner (1). It has also been recognized that the lysosomal degradation pathway of autophagy is used to degrade microorganisms that invade intracellularly (2, 3). Indeed, the mutation of autophagy genes increases susceptibility to infection by intracellular pathogens in multicellular organisms ranging from plants to animals (2). In addition to the direct elimination of intracellular microbes, accumulating evidence further reveals that autophagy plays a critical role in innate and adaptive immunity and inflammation (3, 4). For example, autophagy is both an effector mechanism downstream of Toll-like receptor (TLR) activation (5) and a topological inverter device that can bring cytosolic pathogen-associated molecular pattern molecules into the lumen, where they can bind the ligand recognition side of TLRs (4, 6). Autophagy also lowers the basal level of inflammasome activation, which increases interleukin-1β (IL-1β) processing and secretion, by eliminating defunct mitochondria that otherwise represent endogenous sources of inflammasome agonists, such as reactive oxygen species (7). In addition, the role of autophagy in major histocompatibility complex class II (MHC II) presentation of endogenous cytosolic antigens has been well established in several studies (3, 8, 9). Moreover, autophagy-dependent presentation of endogenous antigens plays a part in positive and negative selection of naïve T-cell repertoires in the thymus (10).

The key morphological features of autophagy are endomembranous organelles, called autophagosomes, whose formation is controlled by autophagy-related genes (Atg) and additional cellular factors (2, 11). Autophagosome formation involves three morphological stages: initiation, elongation and closure, and maturation. Briefly, the Atg system includes the Ser/Thr kinases Ulk1 and Ulk2 (Atg1), Beclin 1 (Atg6; a subunit of the class III phosphatidylinositol 3-kinase [PI3K] human vacuolar protein sorting 34 [hVPS34 complexes]), the Atg5-Atg12/Atg16L1 complex, and microtubule-associated protein L chain 3s (LC3s) (multiple Atg8 orthologs), with LC3B as a commonly used marker for the identification of autophagosomes (12). Ulk1/2 and Beclin 1-hVPS34 integrate upstream signals and direct the downstream Atg conjugation cascade, which involves two ubiquitin-like conjugation systems. The first is the Atg5-Atg12 conjugate. Produced by the Atg7 (E1-like) and Atg10 (E2-like) enzymes, it functions as a dimeric complex together with Atg16L1. The second consists of the phosphatidylethanolamine (PE)-conjugated Atg8 homologues, such as LC3, which are produced by the Atg7 and Atg3 (E2-like) enzymes. Lipidated LC3s, in conjunction with other factors, assemble, elongate, and close nascent autophagic organelles. Autophagosomes interact with endosomal and lysosomal organelles to mature into autolysosomes, where sequestered targets are degraded (2, 4).

Orientia tsutsugamushi, the causative agent of scrub typhus, is an obligate intracellular bacterium (13). The bacteria are transmitted from chigger mites to humans, after which *O. tsutsugamushi* invades cells in the dermis, causing an inflammatory lesion called an eschar (14). If not properly treated in the early stage of infection, scrub typhus patients often develop severe pneumonitis, meningitis, and renal failure (15, 16). These diverse pathological changes in multiple organs are mainly due to focal or disseminated multiorgan vasculitis or perivasculitis of small blood vessels. The bacterium infects several types of nonphagocytic cells, such as endothelial cells and fibroblasts, as well as phagocytic macrophages, polymorphonuclear leukocytes (PMNs), and dendritic cells *in vitro* and *in vivo* (17–21). Previously, it was reported that increased autophagosomes were observed in PMNs after infection with *O. tsutsugamushi* (22). The study is one of the first observations of autophagosomes in eukaryotic cells infected with a bacterial pathogen. Interestingly, intact *O. tsutsugamushi* was rarely

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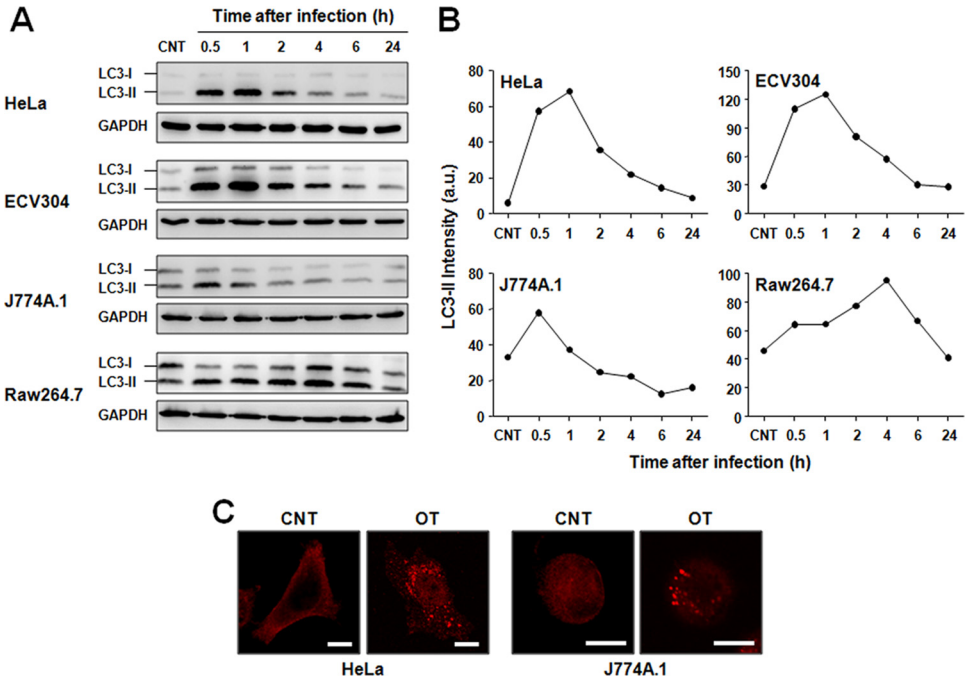

FIG 1 Induction of autophagy by *O. tsutsugamushi* infection. (A) Nonphagocytic cells (HeLa and ECV304) or phagocytic cells (J774A.1 and Raw264.7) were infected with *O. tsutsugamushi* for the indicated times and subjected to immunoblot analysis for LC3-II and for GAPDH as a loading control. (B) The band intensity of LC3-II was measured by densitometric analysis of the immunoblot results (A). a.u., arbitrary units. (C) Induction of cellular autophagy was monitored by confocal microscopy after immunofluorescence staining of indicated cell lines with anti-LC3 antibody after 2 h of infection with *O. tsutsugamushi*. CNT, uninfected control. Bars, 5 μm.

found in the induced autophagosomes of infected PMNs (22). Another study showed that gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α)-stimulated endothelial cells were capable of killing *Rickettsia*, a sister clade of *O. tsutsugamushi*, in association with autophagy (23). Recently, it has been proposed that pathogenic *Rickettsia* may restrict cellular autophagy and thereby contribute to bacterial pathogenesis and cellular tropism (24, 25).

Currently, there is limited knowledge of the role of cellular autophagy in *O. tsutsugamushi* infection. Therefore, we explored the possible involvement of autophagosomes during *O. tsutsugamushi* infection in phagocytic and nonphagocytic cells. Our results demonstrate that live *O. tsutsugamushi* is capable of actively escaping from host autophagosomes even though bacterial infection effectively induces the innate defense system. In addition, chemical modulators of cellular autophagy or genetic knockout of the *atg3* gene did not significantly affect the intracellular growth of *O. tsutsugamushi*.

MATERIALS AND METHODS

Cell culture. HeLa cells (ATCC CCL-2; American Type Culture Collection, Manassas, VA), L929 cells (ATCC NCTC929), ECV304, an endothelial-like cell line (26), J774A.1 (ATCC TIB-67), and Raw264.7 (ATCC TIB-71) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Daegu, South Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Welgene), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD) at 37°C in 5% CO_2. The $Atg3^{+/+}$ and $Atg3^{-/-}$ mouse embryonic fibroblast (MEF) cell lines immortalized by human papillomavirus 16 E6/E7 were kindly provided by Jae U. Jung (University of Southern California).

Preparation and infection of *O. tsutsugamushi*. The Boryong strain of *O. tsutsugamushi* was purified using a modified Percoll gradient purification method (21). *O. tsutsugamushi* was propagated in L929 cells. At 3 to 4 days postinfection, infectivity was determined using an indirect immunofluorescence assay (see below). When an infection rate of >90% was achieved, the cells were harvested by centrifugation at 6,000 × *g* for 20 min. The cell pellet was resuspended with 6.5 ml of Tris-sucrose (TS) buffer (33 mM Tris-Cl [pH 7.4] and 0.25 M sucrose), and the cells were homogenized using 100 strokes of a Polytron homogenizer (Wheaton, Inc., Millville, NJ) followed by centrifugation at 200 × *g* for 5 min. The supernatant was then mixed with 40% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in TS buffer and centrifuged at 25,000 × *g* for 60 min. The bacterial band was collected and centrifuged at 77,000 × *g* for 30 min. The bacterial pellet was washed 3 times in TS buffer, resuspended in DMEM, and stored in liquid nitrogen until use. The infectivity titer of the inoculum was determined as previously described (17), with minor modifications. Infected-cell-counting units (ICU) were calculated as follows: [(total number of cells used for infection) × (percentage of infected cells) × (dilution of *O. tsutsugamushi* suspension)]/100 (17). For infection assays, 2.5×10^6 and 7×10^7 ICU of *O. tsutsugamushi* were used to infect cells cultured in 24-well plates and 100-mm dishes, respectively. UV-inactivated *O. tsutsugamushi* cells were prepared by exposing bacteria to a 30-W UV lamp for 10 min at a distance of 20 cm with gentle shaking.

Antibodies and reagents. Anti-LC3 (NB100-2220; Novus Biologicals, Littleton, CO), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (6C5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-lysosome-associated protein 2 (LAMP2; BD Biosciences), and horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology) were used for immunoblot analysis or immunofluorescence assay. Alexa Fluor 488-, Alexa Fluor 594-, or Alexa Fluor 633-conjugated antihuman, anti-mouse, and antirabbit antibodies were purchased from Molecular Probes (Invitrogen) and used in the immunofluorescence assays. Tetracycline,

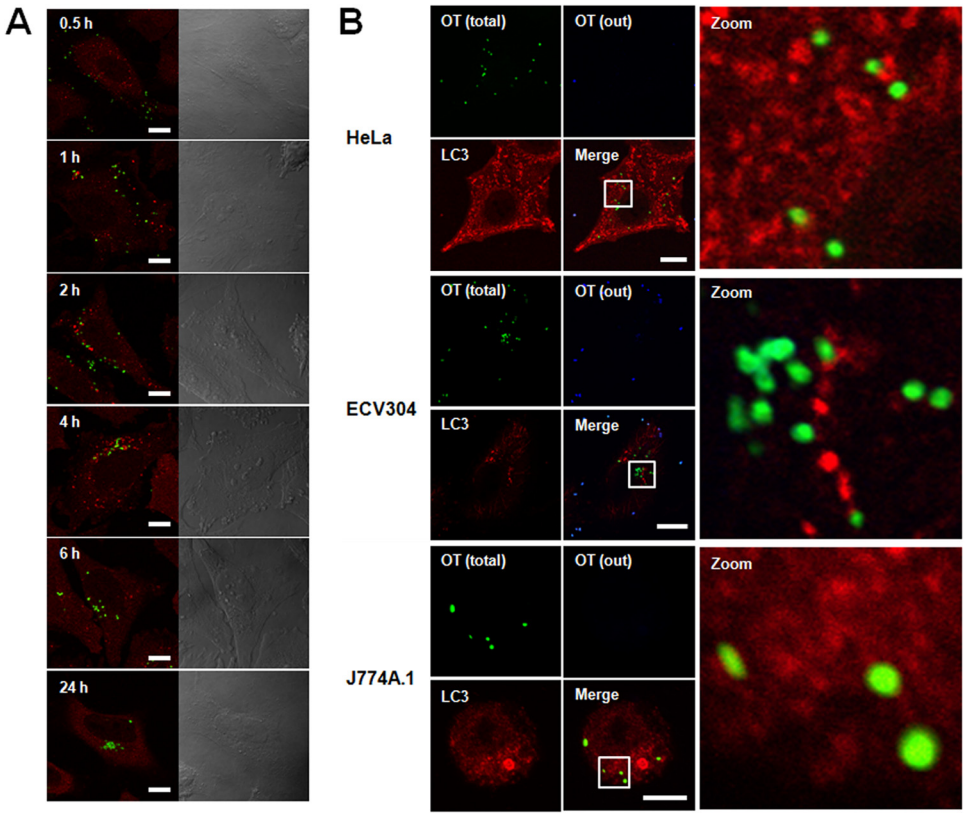

FIG 2 Escape of *O. tsutsugamushi* from autophagy. (A) HeLa cells were infected with *O. tsutsugamushi* for the indicated times and subjected to confocal microscopic analysis after differential immunofluorescent staining of *O. tsutsugamushi* (internal bacteria, green; extracellular bacteria, bright blue [see Materials and Methods]) and autophagosomes (red) stained with anti-LC3 antibody. (B) Localization of autophagosomes (LC3, red) and intracellular *O. tsutsugamushi* (green) was analyzed by confocal microscopy after differential immunofluorescent staining of *O. tsutsugamushi* (internal bacteria, green; extracellular bacteria, bright blue) after 2 h of infection in nonphagocytic cells (HeLa and ECV304) or phagocytic J774A.1 cells. The white inset boxes indicate the regions of the merged images that are shown at a higher magnification on the right (Zoom). OT, *Orientia tsutsugamushi*. Bars, 5 μm.

3-methyladenine (3MA), and rapamycin were obtained from Sigma Chemicals.

Immunoblot analysis. Cells were seeded onto 100-mm tissue culture plates in DMEM with 10% FBS overnight at 37°C. On the following day, cells were either left uninfected or were infected with *O. tsutsugamushi* for the indicated times. After each time point, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and then lysed in 0.5% NP-40 lysis buffer (0.5% NP-40, 20 mM Tris [pH 7.4], 150 mM NaCl, Complete protease inhibitor cocktail [Sigma-Aldrich]). Lysates were centrifuged at 16,000 × *g* for 15 min to pellet insoluble matter. Samples were adjusted for equal protein content and then boiled in SDS sample buffer. Samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA). Immunoblots were developed using the Super Signal West Pico chemiluminescent reagent (Pierce Biotechnology, Rockford, IL).

Immunofluorescence confocal microscopy. Immunofluorescence confocal microscopy was used to visualize *O. tsutsugamushi* (27) and LC3-positive autophagosomes. Cells were cultured on 12-mm-diameter glass coverslips in 24-well plates and inoculated with *O. tsutsugamushi*. Plates were spun at 500 × *g* for 5 min to synchronize bacterial contact with the host cell monolayers and then incubated at 37°C for the indicated times. To discriminate between cell-surface-associated bacteria and intracellular bacteria, infected cells were stained by differential immunofluorescence. First, cells were washed three times with PBS, fixed with 4% paraformaldehyde, and incubated in scrub typhus patients' sera for 1 h, followed by Alexa Fluor 633-conjugated goat anti-human IgG to stain the cell-surface-associated bacteria. Next, cells were permeabilized in a 0.2% Triton X-100 solution for 15 min and incubated with an anti-TSA56 antibody, followed by Alexa Fluor 488-conjugated rabbit anti-mouse IgG to stain intracellular bacteria. Anti-LC3 antibody was used for staining autophagosomes. Cells were observed using an Olympus FV1000 laser confocal microscope (Olympus, Tokyo, Japan) and analyzed using the Fluoview software (Olympus).

RESULTS

***O. tsutsugamushi* induces cellular autophagy but efficiently escapes from the induced autophagosomes.** To determine whether *O. tsutsugamushi* infection induces autophagy *in vitro*, we infected various cell lines, including nonphagocytic (HeLa and ECV304) and phagocytic (J774A.1 and Raw264.7) cells, and monitored autophagy formation by detecting microtubule-associated protein light-chain 3 II (LC3-II) levels (28) and by immunofluorescence confocal microscopy. As shown in Fig. 1A and B, *O. tsutsugamushi* significantly increased endogenous LC3-II protein levels in all cell lines tested, peaking at 0.5 to ~1 h (4 h in Raw264.7 cells) after infection and gradually decreasing thereafter. In addition, the formation of endogenous LC3 punctate structures also significantly increased in both nonphagocytic and phagocytic cells (Fig. 1C and Fig. 2A), indicating that *O. tsutsugamushi* can efficiently induce cellular autophagy in both nonphagocytic and phagocytic cells.

Next, we examined whether the induction of autophagy by *O.*

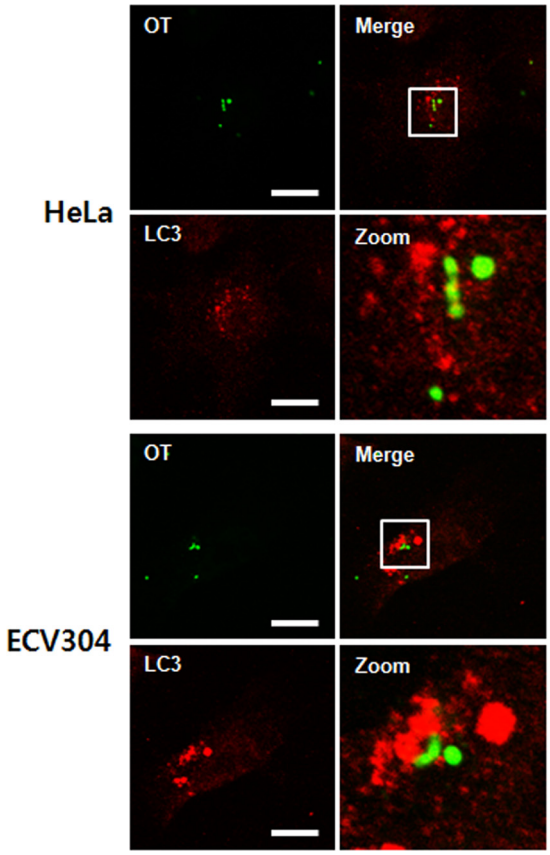

FIG 3 Escape of *O. tsutsugamushi* from autophagy induced by rapamycin treatment. Cells were infected with *O. tsutsugamushi* for 2 h and treated with rapamycin (1 μM) for an additional 2 h. Intracellular localization of *O. tsutsugamushi* (green) and autophagosomes (red) was examined by confocal microscopy after immunofluorescent staining of the infected cells. The white inset boxes indicate the regions of the merged images that are shown at a higher magnification below (Zoom). OT, *Orientia tsutsugamushi*. Bars, 5 μm.

tsutsugamushi infection captures the invading bacteria. Although cellular autophagosome numbers gradually increased up to 2 h after infection, the intracellular bacteria barely colocalized with the LC3-positive autophagosomes throughout the infection in HeLa cells (Fig. 2A and B). The efficient evasion of intracellular *O. tsutsugamushi* from host autophagosomes was also observed in ECV304 cells and J774A.1 macrophages (Fig. 2B). We further tested whether *O. tsutsugamushi* can escape from autophagy induced by rapamycin, which inactivates mTOR and upregulates autophagy (29). Cells were infected with the bacteria for 2 h and then treated with rapamycin for an additional 2 h (Fig. 3). All of the internalized bacteria did not colocalize with autophagosomes, even in the presence of rapamycin, further supporting that *O. tsutsugamushi* can actively escape from induced autophagosomes.

Entrapment of *O. tsutsugamushi* by autophagosomes after antibiotic treatment or UV inactivation. We next questioned whether antibiotic treatment of *O. tsutsugamushi*-infected cells affects bacterial induction of cellular autophagy. Uninfected or HeLa cells infected with *O. tsutsugamushi* for 2 h were treated with tetracycline, which blocks bacterial translation and shows bacteriostatic activity at concentrations of 0.1 to ~0.5 μg/ml under our culture conditions. We found that tetracycline treatment itself did not increase autophagosome formation in either infected or uninfected cells (data not shown). To test whether the inhibition of bacterial translation can induce autophagosomal sequestration of the intracellular bacteria and subsequent lysosomal fusion of the autophagosomes containing the bacteria, we investigated the colocalization of *O. tsutsugamushi* with LC3 and the lysosomal associated membrane glycoprotein-2 (LAMP2) in HeLa cells. LAMP2 is an abundant lysosomal membrane protein that is delivered to phagosomes during their maturation and is required for the fusion of lysosomes with phagosomes (30) and autophagosomes (31). At 6 h after tetracycline treatment (0.3 μg/ml), the internalized bacteria colocalized with LC3-positive autophagosomes in a few infected cells (less than 5%) (Fig. 4A). Moreover, some of the intracellular bacteria colocalized with both autophagosomes and LAMP2-positive lysosomes in the infected cells (Fig. 4A, inset box ["Zoom"] 2). Since the entrapment of *O. tsutsugamushi* by autophagosomes was not efficient and was observed in less than 5% of the infected cells, even after tetracycline treatment, we added rapamycin to the infection medium 6 h after treatment of tetracycline in order to enhance cellular autophagy induction. The incubation of the antibiotic-treated cells with rapamycin for an additional 2 h led to an increase of LC3-positive autophagosomes and colocalization of the infected bacteria with autophagosomes as well as lysosomes in all of the infected cells (Fig. 4B). These indicate that the inhibition of bacterial translation by tetracycline can facilitate autophagosomal entrapment of cytosolic bacteria and subsequent maturation of *O. tsutsugamushi*-containing autophagosomes through lysosomal fusion.

In order to investigate whether inactivation of *O. tsutsugamushi* can facilitate autophagosomal entrapment in phagocytic cells, we analyzed the colocalization of LC3 with UV-inactivated bacteria in Raw264.7 cells. In these experiments, most of the internalized bacteria colocalized with LC3 and LAMP2, whereas live *O. tsutsugamushi* cells efficiently escaped from autophagy (Fig. 5). These data suggest that viability of the intracellular pathogen is critical to its evasion of cellular autophagy in phagocytic cells.

Cellular autophagy does not affect the intracellular growth of *O. tsutsugamushi*. Because *O. tsutsugamushi* infection induces cellular autophagy, but it efficiently escapes from the induced autophagosomes, we further examined whether autophagy affects bacterial growth within host cells. HeLa cells infected with *O. tsutsugamushi* were incubated with increasing doses of 3-methyladenine (3MA), a pharmacological inhibitor of autophagy that blocks class III phosphatidylinositol 3-kinase (32), or rapamycin for 20 h. As shown in Fig. 6, neither the autophagy inhibitor, 3MA (up to 5 mM), nor the autophagy inducer, rapamycin (up to 1 μM), significantly affected bacterial growth within the host cells. The chemical concentrations used were the maximum ranges that were not toxic to HeLa cells, as no morphological and viability changes were observed within 24 h (data not shown).

To further confirm autophagy has no effect on bacterial growth, we employed *atg3*-knockout mouse embryonic fibroblasts ($Atg3^{-/-}$ MEFs), which are defective in autophagosome formation (33). As expected, when $Atg3^{-/-}$ MEFs were infected with *O. tsutsugamushi*, autophagosomes were barely detectable throughout the infection, whereas bacterial infection actively induced cellular autophagy in $Atg3^{+/+}$ MEFs (Fig. 7A). Comparing the levels of bacterial growth at 24 h and 48 h after infection with *O. tsutsugamushi*, the numbers of infected bacteria per cell were highly similar in both MEF lines (Fig. 7B and C). This result pro-

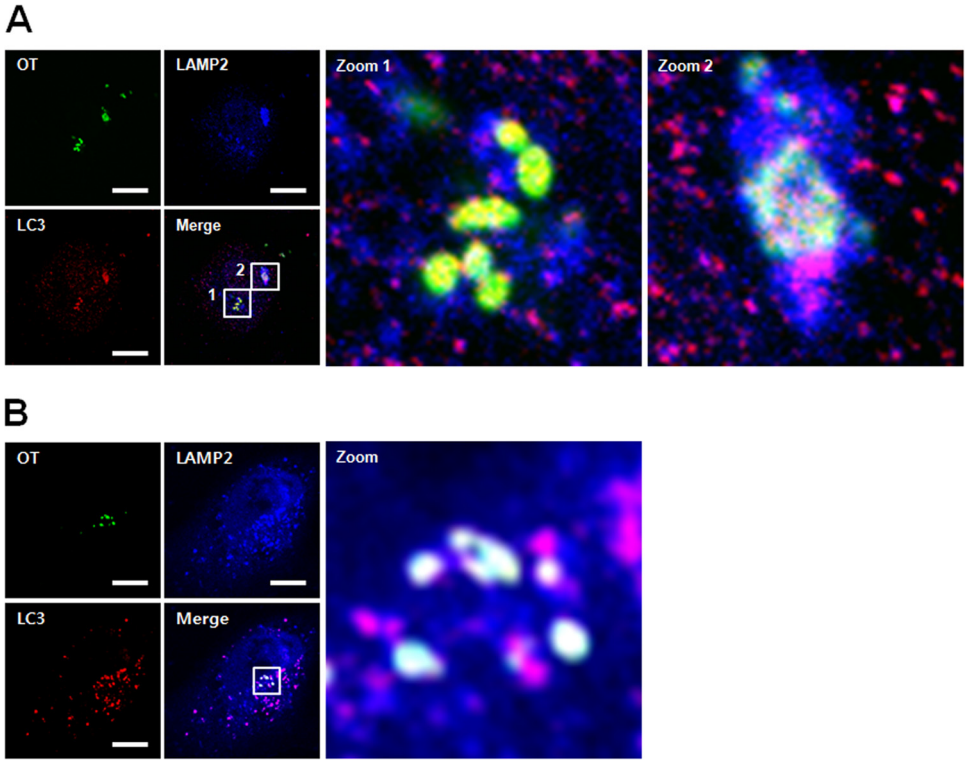

FIG 4 Autophagic entrapment of *O. tsutsugamushi* after tetracycline treatment. (A) HeLa cells were infected with *O. tsutsugamushi* for 2 h and further treated with tetracycline (0.3 μg/ml) for 6 h. Colocalization of intracellular *O. tsutsugamushi* (green), autophagosomes (LC3, red), and LAMP2 (blue), a lysosomal marker, was analyzed by confocal microscopy after immunofluorescent staining. (B) HeLa cells infected with *O. tsutsugamushi* were treated with tetracycline (0.3 μg/ml) for 6 h and further incubated with rapamycin (1 μM) for an additional 2 h. Colocalization of intracellular *O. tsutsugamushi* (green), autophagosomes (LC3, red), and LAMP2 (blue) were examined by confocal microscopy as in panel A. The white inset boxes indicate the regions of the merged images that are shown at a higher magnification on the right (Zoom). OT, *Orientia tsutsugamushi*. Bars, 5 μm.

vides further evidence that the intracellular growth of *O. tsutsugamushi* is not significantly affected by cellular autophagy, even though the bacteria can induce autophagosome formation during the early phase of infection.

DISCUSSION

Intracellular pathogens have evolved to block autophagic microbicidal defenses and subvert host autophagic responses. They can antagonize autophagy initiation or autophagosomal maturation, evade autophagic recognition, or use components of the autophagy pathway to facilitate their own replication or intracellular survival (2, 34). For example, intracellular bacteria that escape into the cytoplasm, such as *Shigella flexneri* and *Listeria monocytogenes*, use strategies to camouflage themselves to avoid autophagic recognition. The *Shigella* type 3 secretion system (T3SS) effector IcsB competitively binds to its own surface protein required for actin-based motility and *Shigella* targeting to autophagosomes, VirG, thereby preventing the interaction between Atg5 and VirG (35). The *Listeria* protein ActA interacts with cytosolic actin polymerization machinery (ARP2/3, VASP, and actin), which blocks bacterial association with ubiquitin, p62 recruitment, and autophagic targeting (36). Another *Listeria* protein, InlK, interacts with the major vault protein (MVP), the main component of cytoplasmic ribonucleoprotein particles, to disguise intracytosolic bacteria and escape autophagic recognition, leading to an increased survival rate of InlK-overexpressing bacteria compared to InlK-negative bacteria (37). The precise mechanisms of these cloaking behaviors are unknown, but it has been proposed that the recruitment of cellular proteins to the bacterial surface may enable intracellular pathogens to disguise themselves as a host cell organelle (36). In the case of *O. tsutsugamushi*, it efficiently evades autophagic recognition, despite activating cellular autophagy during the early phase of infection. Given that inhibition of bacterial translation by tetracycline treatment abrogates bacterial evasion and enhances bacterial entrapment by autophagosomes, the intracellular pathogen may also be equipped with a bacterial factor or factors that block autophagic recognition. The underlying mechanisms of bacterial evasion from autophagy remain to be elucidated.

Microbial pathogens have evolved not only to antagonize autophagy, but also to exploit its components and functions to enhance intracellular survival, replication, or extracellular release of intracellular pathogens (2, 34). Postulated benefits of host autophagy for microbes include the shielding of bacteria from the endolysosomal pathway via the utilization of autophagosomes as a protective intracellular niche and the enhanced survival or growth of intracellular pathogens through the provision of autophagy-generated nutrients (34). *Anaplasma phagocytophilum* replicates within inclusions enveloped with LC3, but does not colocalize with lysosomes (38). Stimulation of autophagy by rapamycin favored *A. phagocytophilum* infection, and inhibition of the autophagosomal pathway by 3MA did not inhibit *A. phagocytophi-*

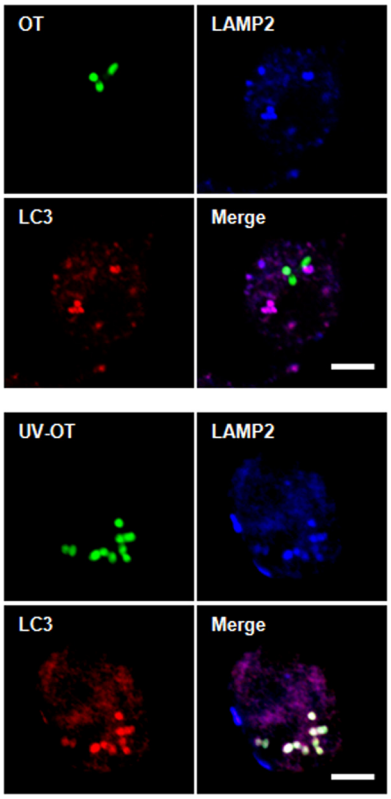

FIG 5 Viability of *O. tsutsugamushi* is required for autophagy evasion in macrophages. Raw264.7 macrophages were incubated with live *O. tsutsugamushi* (upper panels) or UV-inactivated *O. tsutsugamushi* for 2 h and examined under confocal microscopy after immunofluorescent staining of the intracellular bacteria (green), autophagosomes (LC3, red), and lysosomes (LAMP2, blue). OT, *Orientia tsutsugamushi*. Bars, 5 μm.

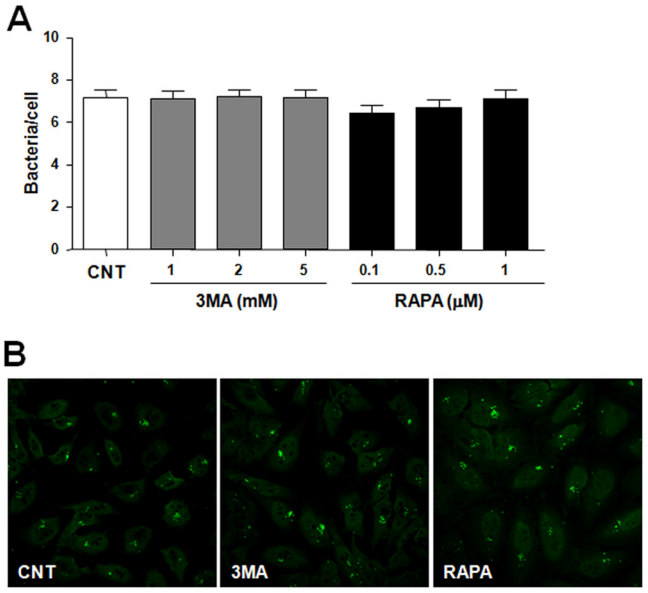

FIG 6 Effect of autophagy modulators on *O. tsutsugamushi* replication. (A) Dose responses of 3MA and rapamycin on *O. tsutsugamushi* replication were examined in HeLa cells. Cells were infected with *O. tsutsugamushi* (~5 bacteria/cell) for 2 h and then treated with the indicated concentrations of 3MA or rapamycin for an additional 20 h. The number of bacterium-infected host cells was enumerated by counting the intracellular bacteria per cell from 100 randomly selected host cells. The bars indicate the means and standard deviations of triplicate experiments. (B) Representative immunofluorescent images of *O. tsutsugamushi* (green)-infected cells are shown. CNT, untreated control.

lum internalization, but reversibly arrested its growth (38). In human epithelial cells infected with *Chlamydia trachomatis*, defective autophagy remarkably enhanced chlamydial growth, suggesting a suppressive effect of the autophagic machinery on bacterial development (39). However, depletion of LC3 in autophagy-deficient cells noticeably reduced chlamydial propagation, suggesting a new function for LC3 in intracellular bacterial pathogenesis, distinct from autophagy (39). Moreover, *Francisella tularensis* reenters endocytic compartments, *Francisella*-containing vacuoles (FCVs), via the autophagy pathway after cytoplasmic replication in macrophages (40). Even though FCV formation does not affect the intracellular survival and replication of *Francisella*, FCVs might promote bacterial egress through exocytosis.

Although several *in vitro* data support the postulates that autophagy may enhance intracellular survival or replication by providing a safe "haven" for a few bacterial species, including *L. monocytogenes*, *Coxiella burnetii*, *Legionella pneumophila*, and *A. phagocytophilum*, whether such mechanisms are important in microbial pathogenesis *in vivo* remains to be explored, and there is no current evidence that autophagy gene deletion in the host attenuates microbial disease (34). Rather, the enhanced pathogenicity of *L. monocytogenes*-infected mice with macrophage-specific deletion of *atg5* (41) and the wild-type levels of replication observed in *L. pneumophila*-infected *Dictyostelium discoideum* lacking autophagy genes (42) suggest that the "microbe-friendly" role of autophagy in microbial replication may not always correlate with the actual role of autophagy in microbial pathogenesis *in vivo* (34). In this study, we did not observe any significant effect of the autophagic chemical inhibitor 3MA or the activator rapamycin on the intracellular growth of *O. tsutsugamushi in vitro*. In addition, bacterial replication in *atg3*-knockout MEFs was comparable to that in wild-type MEFs, strongly supporting that autophagy does not significantly affect the intracellular growth of *O. tsutsugamushi*. Considering that inhibition of bacterial translation by tetracycline-suppressed the active evasion of *O. tsutsugamushi* from autophagy and prominently did so only in the presence of rapamycin, evasion of cellular autophagy might play a critical role in preventing bacterial clearance rather than enhancing bacterial growth. Autophagy is not only involved in the direct killing of intracellular bacteria via lysosome-dependent degradation, but the autophagic delivery of cytoplasmic bacteria to MHC class II loading compartments may also facilitate antigen processing and presentation to antigen-specific $CD4^+$ T cells during tetracycline treatment. Previously, it was shown that IFN-γ- and TNF-α-stimulated endothelial cells were capable of killing *Rickettsia conorii* in association with autophagy (23). The intracellular growth of *O. tsutsugamushi* was also profoundly inhibited in IFN-γ- or TNF-α-treated macrophages (43) and IFN-γ-treated fibroblasts (44). It has been well established that IFN-γ and TNF-α activate autophagy, while the Th2 cytokines IL-4 and IL-13 inhibit autophagy (45). IFN-γ enhances autophagic elimination of intracellular *Mycobacterium tuberculosis*, whereas IL-4 and IL-13 inhibit autophagic control of intracellular mycobacteria (46). Although the role of cytokines in eliminating cytoplasmic *O. tsutsugamushi* via autophagic degradation needs to be confirmed, Th1 cytokines

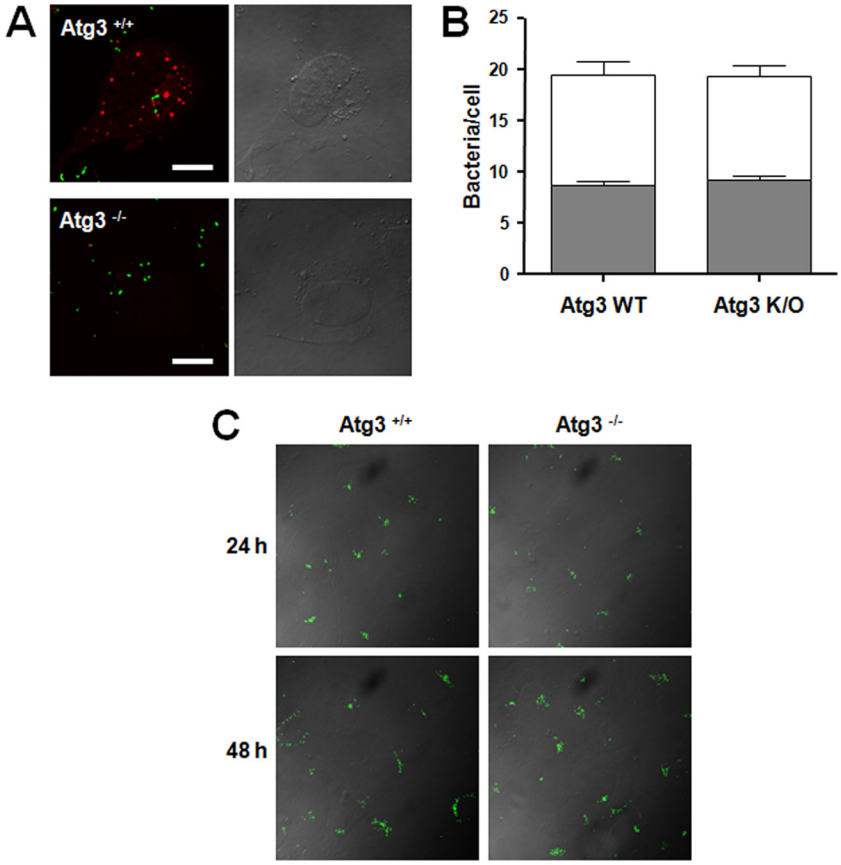

FIG 7 *O. tsutsugamushi* replication in *atg3*-knockout MEFs. (A) Wild-type or *atg3*$^{-/-}$ MEFs were infected with *O. tsutsugamushi*, and the localization of *O. tsutsugamushi* (green) and autophagosomes (red) was analyzed by confocal microscopy after 2 h of infection. Differential interference contrast (DIC) images are shown on the right side. Bars, 5 μm. (B) Intracellular growth of *O. tsutsugamushi* in wild-type MEFs or bacteria per cell from 100 randomly selected MEFs at 24 h (gray bars) and 48 h (white bars) after infection. Growth in *atg3*$^{-/-}$ MEFs was measured by counting the number of intracellular bacteria. The bars indicate the means and standard deviations of triplicate experiments. (C) Representative micrographs of intracellular *O. tsutsugamushi* (green)-infected wild-type or *atg3*$^{-/-}$ MEFs. The immunofluorescent images of bacteria and phase-contrast images of MEFs are overlaid.

may enhance the recognition of cytoplasmic *O. tsutsugamshi* by autophagy and protect the cytosol against bacterial invasion, as observed in cells treated with tetracycline.

In summary, our findings clearly show that *O. tsutsugamushi* actively escapes from cellular autophagy, a cell-autonomous effector mechanism of innate immunity, even though autophagy is efficiently induced during the bacterial infection in both phagocytic and nonphagocytic mammalian cells. The active evasion of autophagy is potentially mediated by bacterial gene expression since the intracellular pathogen is entrapped by autophagosomes and targeted to lysosomes in the presence of tetracycline, which blocks bacterial translation. Finally, we showed that cellular autophagy does not significantly affect the intracellular growth of *O. tsutsugamushi*, as observed in cells treated with chemical modulators of autophagy and in *atg3*-knockout MEFs.

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